Cancer Management and Research

Dovepress

open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

# MiR-34a Inhibits Cell Proliferation and Induces Apoptosis in Human Nasopharyngeal Carcinoma by Targeting lncRNA MCM3AP-AS1

This article was published in the following Dove Press journal:
*Cancer Management and Research*

Piyun Sun
Yuchen Feng
Hui Guo
Rong Li
Peng Yu
Xingguang Zhou
Zhige Pan
Yanyan Liang
Bihan Yu
Yanyi Zheng
Yu Shi
Lingbo Wen
Minmei Wei
Yanhua Chen

Department of Oncology, Liuzhou Hospital of Traditional Chinese Medicine, Liuzhou City, Guangxi Province 545001, People's Republic of China

**Introduction:** MCM3AP-AS1 has been characterized as an oncogenic lncRNA in several types of cancer, while its role in nasopharyngeal carcinoma (NPC) is unknown. This study aimed to investigate the role of MCM3AP-AS1 in NPC.

**Patients and Methods:** Paired NPC tissues and non-tumor tissues were collected from 55 NPC patients. Expression of MCM3AP-AS1 and miR-34a in paired tissues was analyzed by RT-qPCR. Interactions between MCM3AP-AS1 and miR-34a were analyzed by over-expression experiments. The roles of MCM3AP-AS1 and miR-34a in regulating NPC cell proliferation and apoptosis were explored by cell proliferation assay and cell apoptosis assay, respectively.

**Results:** Our bioinformatics analysis showed that MCM3AP-AS1 may be targeted by miR-34a, which is a well-studied tumor suppressor miRNA. In this study, we showed that miR-34a was downregulated and MCM3AP-AS1 was upregulated in NPC. An inverse correlation between the expression of MCM3AP-AS1 and miR-34a was found across NPC tissue samples. High expression level of MCM3AP-AS1 and low levels of miR-34a in NPC tissues predicted the poor survival. In NPC cells, overexpression of MCM3AP-AS1 did not affect the expression of miR34a, while overexpression of miR-34a led to downregulated MCM3AP-AS1. Cell proliferation and apoptosis assay showed that overexpression of miR-34a reduced the enhancing effects of overexpressing MCM3AP-AS1 on cell proliferation and the inhibitory effects on cell apoptosis.

**Conclusion:** MiR-34a inhibits cell proliferation and induces apoptosis in human NPC by targeting MCM3AP-AS1.

**Keywords:** MCM3AP-AS1, miR-34a, nasopharyngeal carcinoma, proliferation, apoptosis

## Introduction

According to the latest GLOBOCAN statistics, nasopharyngeal carcinoma (NPC) affected 129,079 new cases, accounting for 0.7% of all new cancer cases, and caused 72,987 cancer-related deaths, which are 0.8% of all deaths from cancer in 2018 alone.[1] Although NPC is a rare type of cancer, it causes high mortality rate owing to its high malignant nature.[2,3] It has been reported that NPC patients diagnosed at stage I can survive longer than 5 years after the initial diagnosis,[4,5] while only less than 10% of NPC patients are diagnosed at this stage. Once metastasis occurred, the 5-years overall survival rate drops to less than 40%.[6]

Epstein–Barr virus infections and alcohol and tobacco consumptions are the main risk factors for NPC.[7,8] However, these risk factors ae not sufficient for the

Correspondence: Yanhua Chen
Tel +86 772-3357233
Email rp0571@163.com

submit your manuscript | www.dovepress.com
DovePress
http://doi.org/10.2147/CMAR.S245520

occurrence and development of NPC. Studies on the molecular pathogenesis of NPC have revealed a considerable number of molecular players including proteins and non-coding RNAs (ncRNAs) that are involved in this disease.[9,10] Understanding the functionality of the molecular players in this disease provide novel insights into the development of targeted anti-NPC therapy.[9,10] Long ncRNA (lncRNA) MCM3AP-AS1 has been characterized as an oncogenic lncRNA in multiple types of cancer, such as hepatocellular carcinoma, glioblastoma and papillary thyroid cancer,[11–14] while its role in NPC is unknown. Our bioinformatics analysis showed that MCM3AP-AS1 may be targeted by miR-34a, which is a tumor suppressor in NPC.[15] This study was therefore carried out to study the interaction between MCM3AP-AS1 and miR-34a in NPC.

# Patients and Methods

## Patients and Follow-Up

This study included a total of 55 NPC patients (36 males and 19 females, age range of 42 to 66 years old, mean age 54.3 ± 5.3 years old). These patients were enrolled at Liuzhou Hospital of Traditional Chinese Medicine between March 2012 and December 2014. This study was approved by the Ethics Committee of this hospital before enrollment of the patients. All patients provided written informed consent, and that this study was conducted in accordance with the Declaration of Helsinki. Previous treatments and other clinical disorders could also affect gene expression. Therefore, all NPC patients were newly diagnosed cases and no previous history of malignancies were observed. Patients complicated with other clinical disorders or with initiated therapies were excluded. The 55 patients included 12, 14, 18 and 11 cases at AJCC stage I, II, III and IV, respectively. Based on clinical stages and patients health conditions, therapeutic approaches, such as surgical resection, chemotherapy, radiotherapy and the combined therapies were performed. From the day of admission, the 55 patients were followed up for 5 years to monitor their survival. All patients completed the follow-up. Patients died of causes unrelated to NPC were excluded from this study.

## Tissue Collection and NPC Cells

Before therapy, fine needle aspiration (FNA) biopsy was performed on all 55 patients to collect both NPC and adjacent (within 3 cm around tumors) non-tumor tissues. All tissue samples were confirmed by histopathological exam. Fresh tissues were frozen in liquid nitrogen and stored at 37 °C. Two human NPC cell lines C666-1 and 13-9B (SHUNRAN, Shanghai, China) were used. Cell were cultivated following the manufacturer's instructions. Cells were harvested at about 80% confluence for the subsequent experiments.

## Cell Transfections

MCM3AP-AS1 expression vectors were constructed using pcDNA 3.1 vector (Invitrogen) as backbone. Mimic of miR-34a and negative control (NC) miRNA were purchased from Sigma-Aldrich. C666-1 and 13-9B cells were collected and counted. Lipofectamine 2000 reagent (Invitrogen) was used to transfect $10^6$ cells with 10 nM expression vector and (co-transfection)/or 40 nM miRNA with all steps performed following the manufacturer's instructions. Untransfected cells were used as control (C) cells. Empty vector- or NC miRNA-transfected cells were NC cells. Subsequent experiments were performed 48 h later.

## RNA Preparation and RT-qPCR Assay

Total RNAs from tissue samples as well as C666-1 and 13-9B cells were isolated using Trizol reagent (Invitrogen). To harvest miRNA, RNA precipitation and washing steps were performed using 85% ethanol. NanoDrop™ 2000 Spectrophotometer was used to measure RNA concentrations. Genomic DNA was removed after digestion with gDNA eraser (Takara). SSRTIV Reverse Transcriptase kit (Thermo Fisher Scientific) was used to reverse transcribe RNA samples into cDNAs. With cDNA as template, KAPA SYBR® FAST qPCR Master Mix (2X) Kit (KAPA) was used to prepare all qPCR reactions. The expression levels of MCM3AP-AS1 were normalized to the endogenous control GAPDH. The expression levels of mature miR-34a were measured using All-in-One™ miRNA qRT-PCR Reagent Kit (Genecopoeia) with U6 as endogenous control. All PCR reactions were performed in triplicate and $2^{-\Delta\Delta CT}$ method was used to normalize the gene expression levels.

## Cell Proliferation Assay

Cell counting kit (CCK-8, Dojindo, USA) was used to analyze cell proliferation at 48 h after transfection. Each well of a 96-well cell culture plate was filled with 0.1 mL medium containing 4000 cells. Cells were cultivated at 37 °C, followed by the addition of CCK-8 solution to reach a final concentration of 10% at 4 h before cell collection. Cells were collected every 24 h for a total of 4 d. A microplate reader (Bio-Rad, USA) was used to measure OD values at 450 nm.

## Apoptosis Assay

Cells were harvested at 24 h post-transfection, followed by washing with PBS twice and digestion with 0.25% trypsin to prepare single-cell suspensions. Ice-cold 70% ethanol was then used to fix the cells. Staining with propidium iodide (PI) and Annexin-V FITC was performed. Apoptotic cells were detected by FACSCalibur instrument.

## Cell Cycle Analysis

Cells were harvested 72 h after transfection and the cell suspension was then digested. Cells were then fixed with 75% ethanol at 4 °C for 4 h and the supernatant was discarded, followed by incubation with an RNA enzyme containing iodide (PI, 40%, Sigma-Aldrich). Cells were washed with PBS for three times and cell cycle was detected using FACS Calibur (BD Biosciences, USA). Data analysis was conducted through FACS Diva (BD Biosciences, USA). The experiment was repeated three times.

## Colony Formation Assay

A 6-well plate containing solid medium (0.3% agar) was used to cultivate cells with 1000 cells per well. Cells were cultivated at 37 °C in a 5% $CO_2$ incubator for 4 h and images were taken.

## Western Blot Analysis

Cells were lysed with lysis buffer (20 mM KCl, 150 mM NaCl, 1% NP-40, 1% Triton X-100, 50 mM NaF, 50 mM Tris, 1 mM DTT, 1 mM EGTA, 1 x protease inhibitor and 10% glycerol) for 1 h, followed by centrifugation at 4 °C for 30 min. Equal amounts of protein were separated on SDS-PAGE gels and transferred to PVDF membranes. After blocking with 5% non-fat milk, the membranes were incubated with primary antibodies and then with secondary antibodies. Signals were detected using an ECL kit (Bio-Rad)

## Statistical Analysis

Data were expressed as mean ± SEM values of 3 biological replicates. Paired *t* test was used to compare paired tissue samples. ANOVA Tukey's test was used to compare differences among multiple groups. Correlations were analyzed by Pearson's correlation coefficient. The 55 patients were divided into high (n = 28) and low (n = 27) MCM3AP-AS1 or miR-34a level groups based on their median expression levels in NPC tissues as cutoff value. Survival curves were plotted based on K-M method and compared by Log rank test. $P < 0.05$ was considered as statistically significant.

# Results

## The Expression of miR-34a and MCM3AP-AS1 Was Altered in NPC

The differential expression of miR-34a and MCM3AP-AS1 in NPC was determined by measuring their expression levels in paired NPC and non-tumor tissues from the 55 NPC patients included in this study. Compared to non-tumor tissues, significantly lower expression levels of miR-34a were observed in NPC tissues (Figure 1A, $p < 0.001$). In contrast, the expression levels of MCM3AP-AS1 were significantly higher in NPC tissues in comparison to that in non-tumor tissues (Figure 1B, $p < 0.001$)

## The Expression Levels of MCM3AP-AS1 and miR-34a Were Inversely Correlated Across NPC Tissue Samples

Correlation analysis showed that the expression levels of MCM3AP-AS1 were inversely and significantly correlated

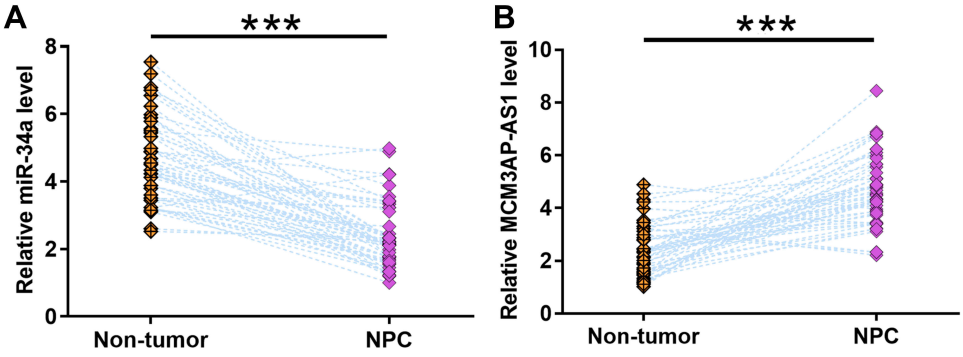


**Figure 1** The expression of miR-34a and MCM3AP-AS1 was altered in NPC. The differential expression of miR-34a (**A**) and MCM3AP-AS1 (**B**) in NPC was analyzed by measuring their expression levels in paired NPC and non-tumor tissues from the 55 NPC patients included in this study. PCR reactions were repeated 3 times and mean values were presented and compared ***$p < 0.001$.

with the expression levels of miR-34a across NPC tissue samples (Figure 2A). In contrast, the correlation between them was not significant across non-tumor tissues (Figure 2B).

## High Expression Levels of MCM3AP-AS1 and Low Expression Levels of miR-34a in NPC Tissues Predicted Poor Survival

Survival analysis was performed following aforementioned method. Compared to patients in high miR-34a group, patients in the low miR-34a group showed obvious lower overall survival rate (Figure 3A). In contrast, patients in high MCM3AP-AS1 level group showed significantly lower overall survival rate in comparison to patients in low MCM3AP-AS1 level group (Figure 3B).

## MiR-34a Targeted MCM3AP-AS1 to Downregulate Its Expression in NPC Cells

Bioinformatics analysis performed using IntaRNA 2.0 showed that MCM3AP-AS1 might be targeted by miR-34a (Figure 4A). To further analyze the interaction between them, C666-1 and 13-9B cells were transfected with either MCM3AP-AS1 overexpression vector or miR-34a mimic. Overexpression of MCM3AP-AS1 and miR-34a was confirmed at 48 h post-transfection (Figure 4B, $p < 0.05$). Compared to C and NC groups, overexpression of miR-34a led to downregulated MCM3AP-AS1 (Figure 4C, $p < 0.05$), while overexpression of MCM3AP-AS1 did not affect miR-34a overexpression (Figure 4D). Overexpression of MCM3AP increased G1/S arrest relative to the control cells (Figure 4E)

## MiR-34a Inhibited NPC Cell Proliferation and Increased Apoptosis Through MCM3AP-AS1

Cell proliferation (Figure 5A) and apoptosis (Figure 5B) assays were performed to study the role of MCM3AP-AS1 and miR-34a in regulating NPC cell proliferation and apoptosis. It was observed that overexpression of

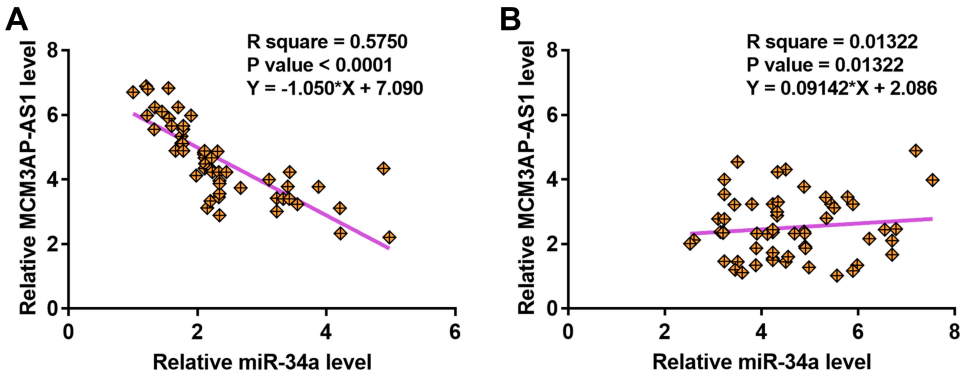


**Figure 2** Expression levels of MCM3AP-AS1 and miR-34a were inversely correlated across NPC tissue samples. Pearson's correlation coefficient was used to analyze the correlations between expression levels of MCM3AP-AS1 and miR-34a across NPC tissues (**A**) and non-tumor tissues (**B**).

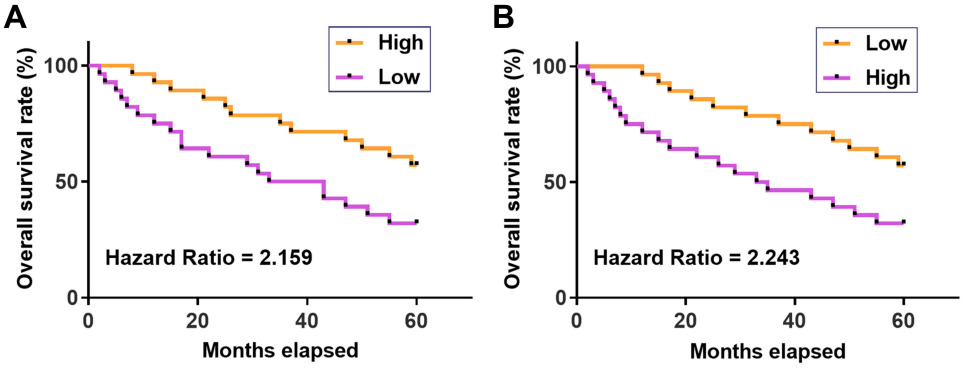


**Figure 3** High expression levels of MCM3AP-AS1 and low expression levels of miR-34a in NPC tissues predicted the poor survival. The 55 patients were divided into high (n = 28) and low (n = 27) miR-34a (**A**) and MCM3AP-AS1 (**B**) level groups based on their median expression level in NPC tissues as cutoff value. Survival curves were plotted based on K-M method and follow-up data. Survival curves were compared by Log rank test.

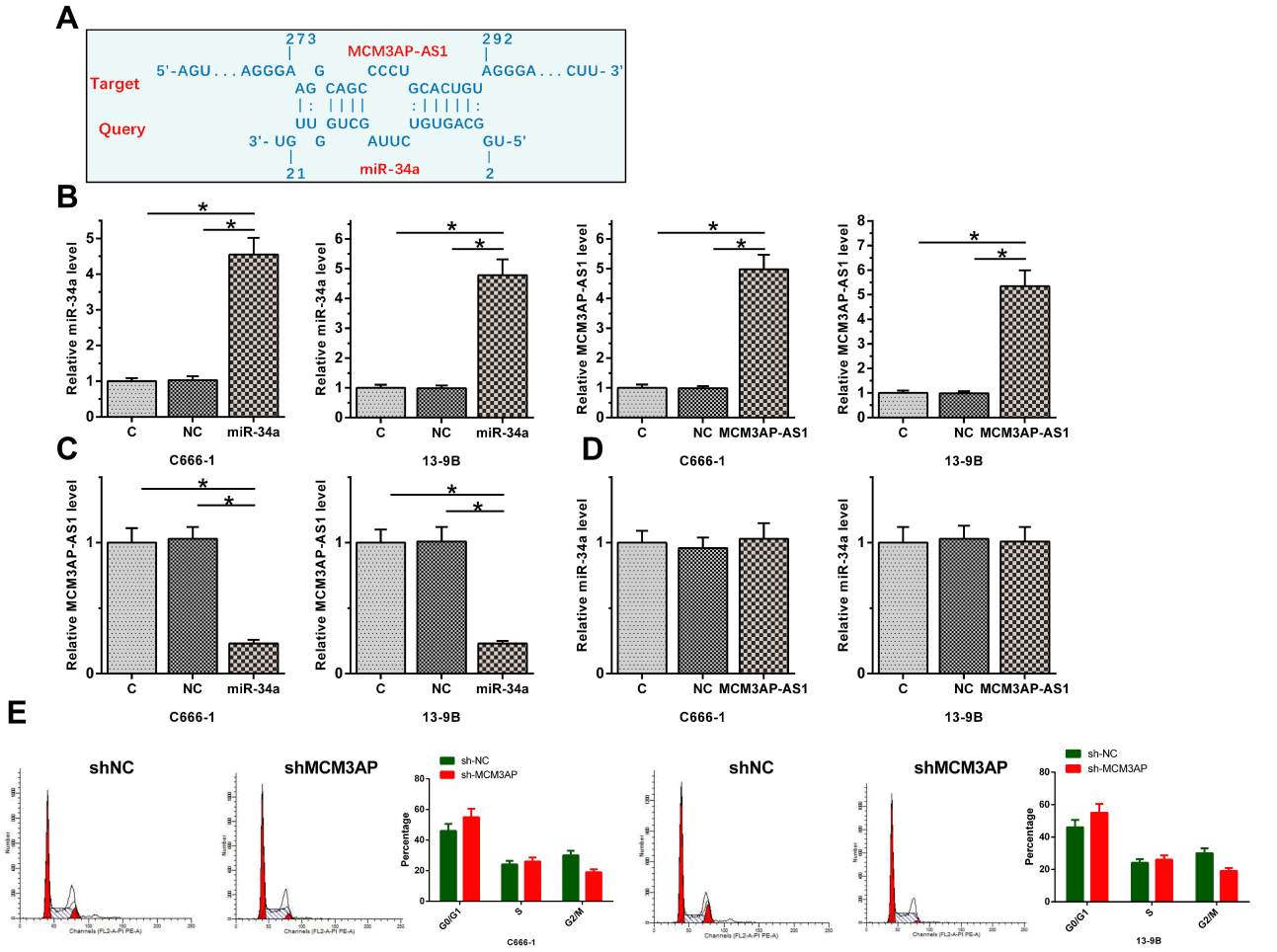


**Figure 4** MiR-34a targeted MCM3AP-AS1 to downregulate its expression in NPC cells. Bioinformatics analysis performed using IntaRNA 2.0[16] showed that MCM3AP-AS1 may be targeted by miR-34a (**A**). C666-1 and 13-9B cells were transfected with either MCM3AP-AS1 overexpression vector or miR-34a mimic and overexpression was confirmed at 48h post-transfection (**B**). The effects of overexpression of miR-34a on MCM3AP-AS1 (**C**) and the effects of overexpression of MCM3AP-AS1 on miR-34a (**D**) were also analyzed by RT-qPCR at 48h post-transfection. C666-1 and 13-9B cells were treated with sh-NC and sh-MCM3AP, and cell cycling was measured by FACS (**E**). All PCR reactions were repeated 3 times and mean values were presented and compared $*p < 0.05$.

MCM3AP-AS1 led to increased cell proliferation and inhibited cell apoptosis ($p < 0.05$). In contrast, miR-34a played an opposite role and reduced the enhancing effects of overexpressing MCM3AP-AS1 on cell proliferation and the inhibitory effects on cell apoptosis ($p < 0.05$). It is worth noting that cell proliferation and apoptotic rates were consistent with the expression levels of MCM3AP-AS1, but not the expression levels of miR-34a (Figure 6A) across transfection groups. Overexpression of MCM3AP increased cell proliferation of C666-1 and 13-9B cells, but the effects were abolished with the transfection of miR-34a (Figure 6B). Furthermore, overexpression of MCM3AP increased the expression of proliferation marker Ki-67, but the expression levels of Ki-67 were reduced with the transfection of miR-34a. Besides, Western blot results showed that overexpression of MCM3AP could downregulate the expression of c-caspase3 and c-caspase9, but the expression levels of c-caspase3 and c-caspase9 were increased with the transfection of miR-34a, which further explained the effect of MCM3AP and miR-34a on the apoptosis of nasopharyngeal carcinoma cells (Figure 6C).

## Discussion

In this study, we investigated the interaction between miR-34a and MCM3AP-AS1 in NPC. We found that MCM3AP-AS1 is upregulated in NPC and can be targeted by miR-34a.

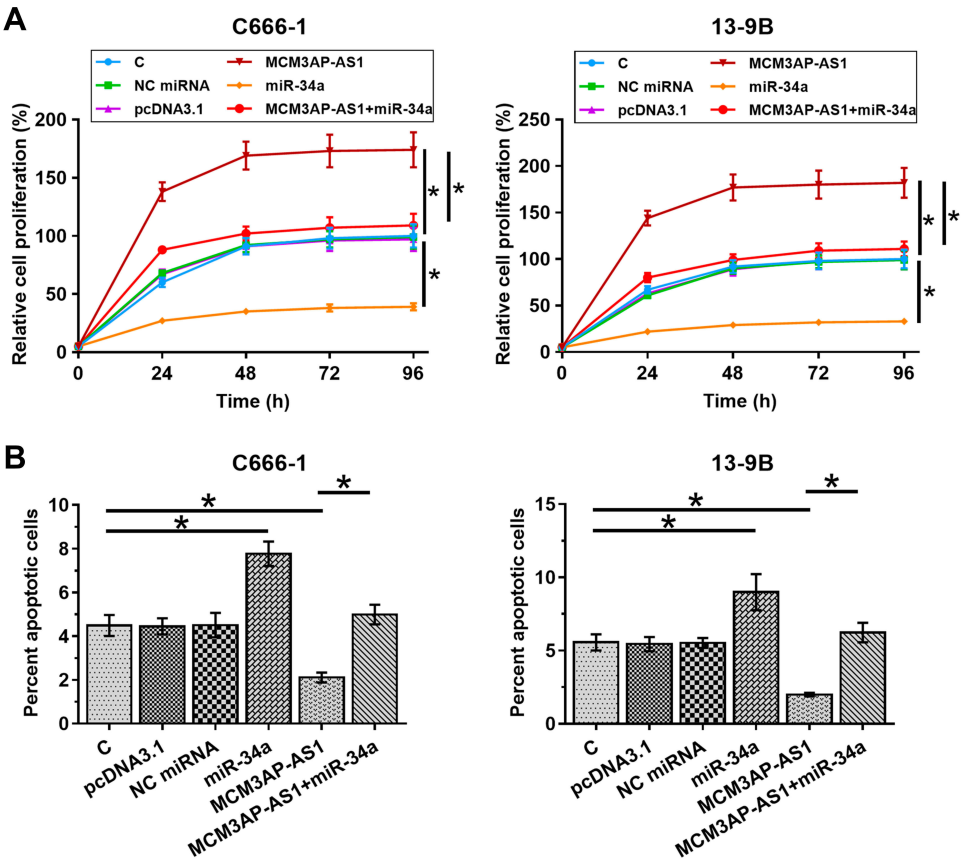


**Figure 5** MiR-34a inhibited NPC cells proliferation and increased apoptosis through MCM3AP-AS1. Cell proliferation (**A**) and apoptosis (**B**) assays were performed to analyze the role of MCM3AP-AS1 and miR-34a in regulating NPC cell proliferation and apoptosis. All PCR reactions were repeated 3 times and mean values were presented and compared *$p < 0.05$.

Previous studies have characterized MCM3AP-AS1 as an oncogenic lncRNA in multiple types of cancer.[11–14] For instance, MCM3AP-AS1 is overexpressed in hepatocellular carcinoma and promotes cancer cell proliferation by regulating the miR-194-5p/FOXA1 axis.[11] MCM3AP-AS1 is also upregulated in papillary thyroid cancer and regulate miR-211-5p/SPARC axis to promote the invasion and proliferation of cancer cells.[12] This study is the first to report the upregulation of MCM3AP-AS1 in NPC. In addition, we also showed that overexpression of MCM3AP-AS1 led to promoted NPC cell proliferation and suppressed cell apoptosis. Therefore, MCM3AP-AS1 is likely an oncogenic lncRNA in NPC.

More than 80% of NPC patients are diagnosed at advanced stages.[4,5] Due to the lack of sensitive and reliable early diagnostic markers, the low diagnostic rate of NPC is unlikely to be significantly improved in near future. Therefore, accurate prognosis of this disease may improve patients' survival by guiding the determination of treatment approaches and the development of individualized post-treatment care system. In this study, we showed that high expression levels of MCM3AP-AS1 and low expression levels of miR-34a were associated with poor survival of NPC patients, indicating that MCM3AP-AS1 and miR-34a might serve as prognostic biomarkers for NPC. However, the accuracy remains to be further tested.

All previous studies of MCM3AP-AS1 focused on its downstream effector.[11–14] The upstream regulators of MCM3AP-AS1 are unknown. In this study, we proved that miR-34a might target MCM3AP-AS1. It is worth noting that the expression levels of MCM3AP-AS1 and miR-34a are only significantly correlated across NPC tissue samples but not non-tumor tissues. Therefore, the targeting of MCM3AP-AS1 by miR-34a is likely NPC-specific.

In conclusion, MCM3AP-AS1 is overexpressed in NPC. MiR-34a may target MCM3AP-AS1 to suppress NPC cell proliferation and promote cancer proliferation.

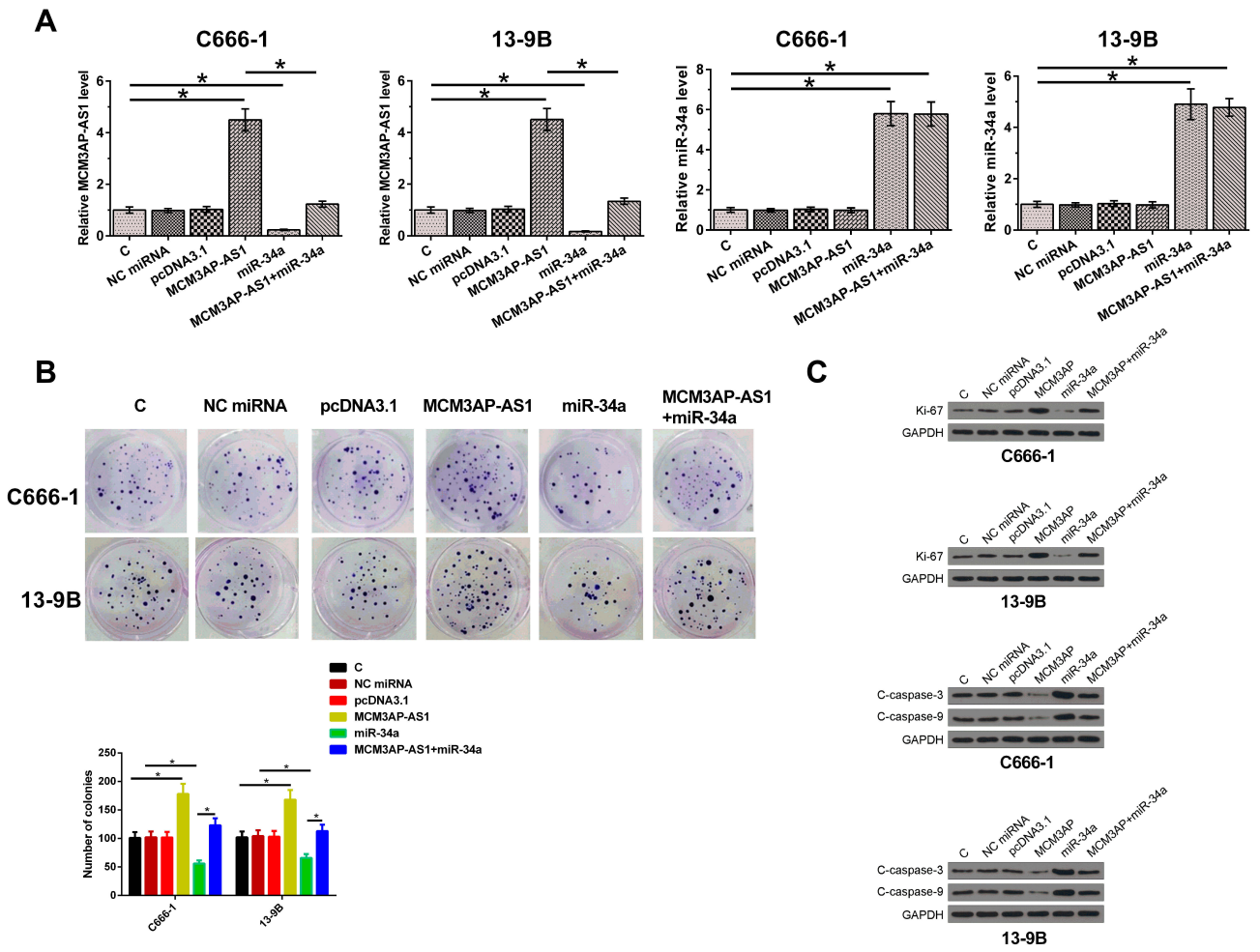


**Figure 6** MCM3AP-AS1 promoted the proliferation and suppressed apoptosis through MCM3AP-AS1 of NPC cells. (**A**) RT-qPCR was performed to measure the expression levels of MCM3AP-AS1 and miR-34a in cells with different transfections. (**B**) Colony formation was used to detect the proliferation of C666-1 and 13-9B cells. (**C**) Western blot analysis was used to detect the level of Ki-67, c-caspase3 and c-caspase9 in C666-1 and 13-9B cells. All PCR reactions were repeated 3 times and mean values were presented and compared $^*p < 0.05$.


## Funding

Not applicable.


## Disclosure

The authors declare that they have no competing interests.

## References


1. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394–424. doi:10.3322/caac.21492
2. Chua MLK, Wee JTS, Hui EP, et al. Nasopharyngeal carcinoma. *Lancet*. 2016;387(10022):1012–1024. doi:10.1016/S0140-6736(15)00055-0
3. Tang LL, Chen WQ, Xue WQ, et al. Global trends in incidence and mortality of nasopharyngeal carcinoma. *Cancer Lett*. 2016;374(1):22–30. doi:10.1016/j.canlet.2016.01.040
4. Sun X, Su S, Chen C, et al. Long-term outcomes of intensity-modulated radiotherapy for 868 patients with nasopharyngeal carcinoma: an analysis of survival and treatment toxicities. *Radiother Oncol*. 2014;110(3):398–403. doi:10.1016/j.radonc.2013.10.020
5. Chan ATC. Nasopharyngeal carcinoma. *Ann Oncol*. 2010;21(suppl_7):vii308–vii312. doi:10.1093/annonc/mdq277
6. Peng H, Chen L, Zhang Y, et al. Survival analysis of patients with advanced-stage nasopharyngeal carcinoma according to the Epstein-Barr virus status. *Oncotarget*. 2016;7(17):24208–24216. doi:10.18632/oncotarget.8144
7. Tsao SW, Tsang CM, Lo KW. Epstein–Barr virus infection and nasopharyngeal carcinoma. *Philos Trans R Soc Lond B Biol Sci*. 2017;372(1732):20160270. doi:10.1098/rstb.2016.0270
8. Omar RMR. Risk factors associated with nasopharyngeal carcinoma in sabah: study protocol of a case control study. *BJMS*. 2018;43.
9. Chan KC, Chan LS, Ip JCY, et al. Therapeutic targeting of CBP/β-catenin signaling reduces cancer stem-like population and synergistically suppresses growth of EBV-positive nasopharyngeal carcinoma cells with cisplatin. *Sci Rep*. 2015;5:9979. doi:10.1038/srep09979
10. Lee KTW, Tan JK, Lam AK, et al. MicroRNAs serving as potential biomarkers and therapeutic targets in nasopharyngeal carcinoma: a critical review. *Crit Rev Oncol Hematol*. 2016;103:1–9. doi:10.1016/j.critrevonc.2016.04.006
11. Wang Y, Yang L, Chen T, et al. A novel lncRNA MCM3AP-AS1 promotes the growth of hepatocellular carcinoma by targeting miR-194-5p/FOXA1 axis. *Mol Cancer*. 2019;18(1):28. doi:10.1186/s12943-019-0957-7

12. Yang C, Zheng J, Xue Y, et al. The effect of MCM3AP-AS1/miR-211/KLF5/AGGF1 Axis regulating glioblastoma angiogenesis. *Front Mol Neurosci*. 2018;10:437. doi:10.3389/fnmol.2017.00437
13. Liang M, Jia J, Chen L, et al. LncRNA MCM3AP-AS1 promotes proliferation and invasion through regulating miR-211-5p/SPARC axis in papillary thyroid cancer. *Endocrine*. 2019;65(2):318–326. doi:10.1007/s12020-019-01939-4
14. Zhang H, Luo C, Zhang G. LncRNA MCM3AP-AS1 regulates epidermal growth factor receptor and autophagy to promote hepatocellular carcinoma metastasis by interacting with miR-455. *DNA Cell Biol*. 2019;38(8):857–864. doi:10.1089/dna.2019.4770
15. Long Z, Wang B, Tao D, et al. Hypofractionated radiotherapy induces miR-34a expression and enhances apoptosis in human nasopharyngeal carcinoma cells. *Int J Mol Med*. 2014;34(5):1388–1394. doi:10.3892/ijmm.2014.1937
16. Mann M, Wright PR, Backofen R. IntaRNA 2.0: enhanced and customizable prediction of RNA–RNA interactions. *Nucleic Acids Res*. 2017;45(W1):W435–W439. doi:10.1093/nar/gkx279